MAMMALIAN ~~*elk*~~ POTASSIUM CHANNEL GENES

FIELD OF THE INVENTION

The present invention relates to the isolation, cloning and sequencing of genes and the encoded proteins for voltage-gated potassium channels (K channels). More particularly, the invention is directed to the isolation, cloning and sequencing of *eag* and *eag-like-K+* channel genes (*elk*).

The availability of these genes provide the tools for further reasearch into the physiobiological characteristics of the various genes and proteins for K channels; the development of medicines which are effective for treating the specific disease conditions arising from mutations or defects in the specific K channels and the screening of drugs to ensure that the elk potassium channels will not be blocked. The availability of the *elk* gene/elk protein ensures that drugs screened for the treatment of other diseases will not affect the physiological functions controlled by the *eag* and *elk* potassium channel gene.

BACKGROUND OF THE INVENTION

Potassium channels (K channels) play a major role in a wide range of cellular functions, including electrical excitability and synaptic plasticity in neurons. K channels are involved in determining and stabilizing the resting membrane potential, the re-polarization of action potentials, control of firing patterns and modulation of neurotransmitter release of nerve cells. Some of the functions controlled are basic, such as the setting of the resting potential; and some of the functions are very specific, such as defining the interval between spikes of endogenously beating cells. These

functions are involved in a number of physiological processes, including regulation of heartbeat, dilation of arteries, release of insulin, excitability of nerve cells, and regulation of renal electrolyte transport. Potassium channels are thus found in a wide variety of animal cells such as nervous, muscular, glandular, immune, reproductive, and epithelial tissue. These channels allow the flow of potassium ions in and/or out of the cell under certain conditions. For example, the outward flow of potassium ions upon opening of these channels makes the interior of the cell more negative, counteracting de-polarizing voltages applied to the cell. These channels are regulated, e.g., by calcium sensitivity, voltage-gating, second messengers, extracellular ligands, and ATP-sensitivity. It is important for the potassium channels to be operating properly to maintain healthy functions in the body. It is, therefore important that medicines for the treatment of diseases unrelated to the function of the ion channels do not adversely affect the proper function of these channels.

It is known that a variety of genes encode the proteins of the K channels with diverse electrophysiological and pharmacological properties. H. Terlau et al., Pflughers Arch., (1996) 432:301-312. The research is ongoing to determine the number of the genes involved and the characteristics and functions of the proteins which the genes encode.

Based on recent molecular cloning studies, a hierarchical classification scheme has been developed. Sodium, calcium and potassium channels belong to a 'superfamily' of ion channels.

Structurally, K channels can also be grouped into

three main classes: six-transmembrane (6TM), four-transmembrane (4TM) and two-transmembrane (2TM) subunits. The 6TM superfamily may be further divided into six families, among which are the voltage-gated, KQT-like, *EAG*-like, Slo-like, cyclic nucleotide-gated and "SK" channels. Each of the families may be further subdivided into "subfamilies". For example, in the voltage-gated family, there are four subfamilies: Shaker, Shab, Shaw and Shal, corresponding to Kv1, Kv2, Kv3 and Kv4, respectively. Between subfamilies of the 6TM family, the observed amino acid sequence homology is about 40%. A higher degree of homology, 50%-70%, is shared among members of a single subfamily of the 6TM family. This is true intra or inter species. Whereas, in the 2TM and 4TM families, much less conservation is observed across species. A schematic of the classification scheme is shown in Fig.1. A. Wei et al., supra.

Potassium channels are typically formed by four alpha subunits, and can be homomeric (made of identical alpha subunits) or heteromeric (made of two or more distinct types of alpha subunits). In addition, potassium channels made from Kv, KQT and Slo subunits have often been found to contain additional, structurally distinct auxiliary, or beta, subunits. These subunits do not form potassium channels themselves, but instead they act as auxiliary subunits to modify the functional properties of channels formed by alpha subunits. For example, the Kv beta subunits are cytoplasmic and are known to increase the surface expression of Kv channels and/or modify inactivation kinetics of the channel (Heinemann et al. J. Physiol 493:625-633 (1996); Shi et al., Neuron 16(4):843-852 (1996)). In another example, the KQT family beta subunit, minK, primarily changes activation kinetics(Sanguinetti et al., Nature 384:8O-83 (1996)).

Voltage-gated K channels are encoded by two groups: the extended Shaker family including the Ca^{2+}-activated SK and BK channels and those ion channels with a cyclic nucleotide-binding domain. The latter include the cyclic nucleotide-gated channels, the plant inward-rectifying channels and the *ether-a-go-go* (EAG) channels. H. Terlau et al., J. Physiol., (1997) 502.3:537-543. The *eag*-like family comprise three related voltage-gated K channel genes. The first of the family, the *eag gene*, was initially identified in *Drosophila*. J. Warmke *et al.*, Science (1991) 252:1560-1562. Two related *Drosophila* genes were subsequently described: the *eag*-related-gene (*erg*) and the *eag*-like-K^+ channel gene (*elk*). Warmke and Ganetzky, PNAS, USA (1994) 91: 3438-3442. Mammalian homologues of the *eag* genes have been identified and the potassium channels that they encode have been expressed and characterized. Warmke and Ganetzky (1994), supra; J. Ludwig *et al.*, EMBO J. (1994) 13:4451-4458; G.A. Robertson *et al.*, Neuropharmacol. (1996) 35:841-850; and W. Shi *et al.*, J Neurosci. (1997) 17:9423-9432.

Up to late 1997, only a single representative of the *erg* family, human *erg* (*Herg*) was found. There is considerable interest in the *erg* gene because mutations were shown to underlie one form of a human genetic disease known as long QT (LQT) syndrome, which gives rise to cardiac arrhythmia and an increase incidence of sudden death.

In *Xenopus* oocytes, *Herg* forms voltage-sensitive K channels with a distinctive inactivation mechanism that attenuates efflux of K+ during depolarization (Sanguinetti et al., Cell (1995) 81, 299-307. The *erg* channels most closely resemble those of native cardiac I_{Kr} channels. Through tissue distribution studies, it was found that the *Herg* gene is expressed abundantly in the nervous system and heart muscle.

Up to the present three members of the mammalian *erg* family have been cloned and sequenced by the inventors and reported in W. Shi et al, J. Neurosci. (1997) 17:9423-9432.

The rat *eag*1 has also been identified, cloned and sequenced. No other *eag* gene has been identified, or cloned. To date, no mammalian homologue of the *Drosophila elk* gene has been described, nor have the biophysical properties of any *elk* channel been characterized.

OBJECTIVES OF THE INVENTION

It is an objective of this invention to isolate, clone, and sequence further *eag* and *elk* genes and to use the nucleic acid sequences obtained to deduce the amino acid sequence of the proteins encoded by further *eag* and *elk* genes.

It is another objective of this invention to isolate, clone, and sequence other genes in the *eag*-like family and to use the nucleic acid sequences obtained to deduce the sequence of the protein encoded by other *eag*-like genes.

It is another objective to determine the biophysical and pharmacological properties of the channels encoded.

It is a further objective to determine the distribution pattern of the mRNA of all known *eag*-like genes in sympathetic ganglia and sciatic nerve.

SUMMARY OF THE INVENTION

Three new members of the mammalian *eag*-like gene family are identified using rat sympathetic ganglia tissue.

The *elk*1 gene has been isolated, cloned and the complete coding sequence (SEQ ID NO:1, Fig. 3) identified. The gene has been expressed in *Xenopus* oocytes and the deduced amino acid sequence (SEQ ID NO:2, Fig 2) of the elk1 protein

was also determined. The biophysical and pharmacological properties of the *elk*1 encoded channel has also been analyzed. The *elk*2 gene was also isolated, cloned and sequenced (SEQ ID NO:13) and the amino acid sequence (SEQ ID NO:3) of the protein deduced.

Further, another member of the *eag* family, the *eag*2 gene was isolated, cloned and sequenced (SEQ ID NO:14) and its deduced amino acid sequence (SEQ ID NO:4) determined.

In addition, the mRNA distribution pattern in rat sympathetic ganglia and sciatic nerve of all known *eag* genes was obtained using three sympathetic ganglia: one paravertebral ganglia, the superior cervical ganglia (SCG); and two prevertebral ganglia, the coeliac ganglia (CG) and the superior mesenteric ganglia (SMG).

The results indicate that The *elk*1 gene is expressed in both prevertebral and paravertebral ganglia at approximately equal levels. In contrast, the *elk*2 gene is not expressed at detectable levels in sympathetic ganglia, although it is robustly expressed in the brain. Both *eag* genes are expressed in sympathetic ganglia. The *eag*1 gene is expressed at high levels in all three sympathetic ganglia. The *eag*2 gene is only expressed at significant levels in the prevertebral ganglia, the CG and SMG.

In addition to neurons, sympathetic ganglia also contain glial cells (known as satellite cells and developmentally related to Schwann cells). To determine whether gene expression in ganglia could be due to expression in glial cells, *EAG* gene expression was examined in sciatic nerve, which contains a relatively pure population of Schwann cells. Two of the seven *EAG* genes tested , *elk1* and *erg1*, are expressed in sciatic nerve (Fig. 4B).

A summary of *EAG* gene expression in sympathetic ganglia and sciatic nerve is shown in Table 1. At least six *EAG* genes are expressed in the sympathetic ganglia and all seven *EAG* genes are expressed in at least one neuronal tissue. Two genes, *eag2* and *erg2*, are differentially expressed in sympathetic ganglia. Both genes are expressed at higher levels in prevertebral ganglia than paravertebral ganglia. The housekeeping gene cyclophilin was expressed at significantly lower levels in sciatic nerve RNA than in ganglia or brain RNA (Fig. 4B). It is possible, therefore, that mRNA levels, as a fraction of total RNA, are significantly lower in sciatic nerve than in ganglia or brain. For this reason, the relative level of *elk1* and *erg1* expression in sciatic nerve described in Table 1 may be an underestimate in comparisons between sciatic nerve and ganglia or brain.

Utilizing the information obtained from rat *elk1*, *elk2* and *eag2* the corresponding human genes can be obtained and the protein encoded thereby expressed.

The present invention thus provides isolated nucleotides from rat and human tissue encoding a polypeptide forming a potassium channel. The polypeptide is selected from the group consisting of elk1, elk2 and eag2. The elk1 polypeptide forms a channel having the characteristic of a slowly activated outward rectifier, with an activation constant of about 100 ms at 0 mV with a threshold of -40 mV. The eag-like K channels also exhibits no apparent inactivation during de-polarizing voltage steps of up to several seconds.

The elk1 channel is characterized by having a activation time constant of 676 ± 37 ms at 0 mV with a threshold of activation at -40 mV and a deactivation rate of

111 ± 7 ms at -50 mV at about pH 7. Based on our observations, the activation/deactivation rate for elk1 is dependent on the pH of the solvent. However, for elk2 and eag2, the activation/deactivation rate is not as pH dependent.

The polypeptide elk1 has a molecular weight of between 100 kDa to 150 kDa, about 123 kDa. The polypeptides elk2 and eag2 have a molecular weight similar to that of elk1 of between 100 kDa to 150 kDa.

The nucleic acids encoding elk1, elk2, or eag2 are amplified by primers that selectively hybridize under stringent conditions to sequences that hybridize to the primers. For *elk*1, the primers are SEQ ID NO:5 and SEQ ID NO:6: for *elk*2, the primers are SEQ ID NOS:9 and 10 and for *eag*2, the primers are SEQ ID NOS:5 and 6. To obtain the equivalent human *elk*1, *elk*2 or *eag*2, the primers may be selected by a comparison of the rat *elk*1, *elk*2 or *eag*2 nucleic acid sequences respectively with the human EST sequences listed in Genbank using the BLAST or PILEUP programs. Sequences from the list of human EST which shows the highest homology with a region of each of the rat sequences for *elk*1 *elk*2 or *eag*2 are obtained. From the EST sequences obtained, nucleotides of between 17 to 25 base pairs with at least 90% homology between the rat and human sequences are selected for use as primers.

In another aspect, the present invention provides an antibody that selectively binds to the polypeptide elk1 elk2 or eag2 described above.

The present invention further provides expression factors comprising the nucleic acid sequences encoding *elk*1, *elk*2 or *eag*2.

The present invention further provides host cells transfected with the expression vectors described above.

In another aspect, the present invention provides a method of drug screening to ensure that the compounds identified for use as drugs for other diseases do not have a deleterious effect on the function of the elk1, elk2 or eag2 potassium channels. The method comprises the steps of: (a) contacting the compound with a eukaryotic host cell or cell membrane in which has been expressed a polypeptide comprising an alpha subunit of a potassium channel selected from the group consisting of elk1, elk2 and eag2, the potassium channel having the characteristic of a slowly activated outward rectifier; and (b) determining the functional effect of the compound upon the cell or cell membrane expressing the potassium channel. One example of the cell or cell membrane expressing the potassium channels are *Xenopus* oocytes or the membranes thereof.

In one embodiment, the functional effect is determined by measuring changes in current or voltage. In another embodiment, the polypeptide monomer polypeptide is recombinant. In another embodiment, the potassium channel is homomeric or heteromeric.

In another embodiment, the present invention provides a method of detecting the presence of elk1, elk2 or eag2 in mammalian tissue, the method comprising the steps of: (i) isolating a biological sample; (ii) contacting the biological sample with a reagent that selectively associates with elk1, elk2 or eag2; and, (iii) detecting the level of reagent that selectively associates with the sample. The reagent is selected from the group consisting of: antibodies specific for elk1, elk2 or eag2, oligonucleotide primers specific for *elk*1,

elk2 or eag2, and for *elk1, elk2 or eag2* nucleic acid probes. The mammalian tissue may be from rat or human.

The present invention also provides, in a computer system, a method of screening for mutations of *elk1, elk2* or *eag2* genes, the method comprising the steps of: (i) entering into the computer system a first nucleic acid sequence encoding an slowly activated outward rectifier potassium channel protein having a nucleic acid sequence of SEQ ID No:1, or comprising a nucleic acid sequence SEQ ID No:13 or SEQ ID No:14; and conservatively modified versions thereof; (ii) comparing the first nucleic acid sequence with a second nucleic acid sequence having substantial identity to the first nucleic acid sequence; and (iii) identifying nucleotide differences between the first and second nucleic acid sequences.

The second nucleic acid sequence may be associated with a disease state. Or, the computer readable substrate comprises the first and the second nucleic acid sequences.

The present invention further provides, in a computer system, a method for identifying a three-dimensional structure of elk1, elk2 or EAG2 polypeptides, the method comprising the steps of: (i) entering into the computer system an amino acid sequence of at least 10 amino acids of elk1, elk2 or eag2 or at least 30 nucleotides of a gene encoding the elk1, elk2 or eag2 polypeptide, the polypeptide having an amino acid sequence of SEQ ID No: 2, or comprising the partial amino acid sequence SEQ ID No:3 or SEQ ID No:4 and conservatively modified versions thereof; and (ii) generating a three-dimensional structure of the polypeptide encoded by the amino acid sequence.

In one embodiment, the amino acid sequence is a

primary structure and wherein said generating step includes the steps of: (i) forming a secondary structure from said primary structure using energy terms determined by the primary structure; and (ii) forming a tertiary structure from said secondary structure using energy terms determined by said secondary structure. In another embodiment, the generating step includes the step of forming a quaternary structure from said tertiary structure using anisotrophic terms determined by the tertiary structure. In another embodiment, the methods further comprises the step of identifying regions of the three-dimensional structure of the protein that bind to ligands and using the regions to identify ligands that bind to the protein. In another embodiment, a computer readable substrate comprises the three dimensional structure of the polypeptide monomer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of the classification of the K channel genes.

Fig. 2 is an alignment of the *elk*1, *erg*1 and *eag*1 deduced amino acid sequences. Identical residues are double underlined and similar residues are single underlined. The six hydrophobic domains (S1-6), and the pore (P) and the putative cyclic nucleotide binding domain (cNBD) are overlined.

Fig. 3 is the cDNA sequence of the *elk*1 gene (SEQ ID NO.1).

Figs. 4A-4H show the electrophysiological characteristics of the rat *elk*1 expressed in *Xenopus* oocytes.

Fig. 4A shows the voltage-clamp recordings of *elk*1 currents with voltage steps over the range of -60 to +50 mV

from a holding potential of -70 mV. Tail currents were recorded at -50 mV.

Fig. 4B show the normalized conductance-voltage curve of the elk1 channel. Conductance of the channel was calculated as the current measured at the end of a 2 second depolarizing step divided by the driving force (EK = -75 mV). Data points are averages from 7 oocytes and were fitted with the equation:

$$G/Gmax = 1/(1 + \exp((V-Vn)/kn))$$

where Vn, the midpoint for activation, was 9.3 ± 0.4 mV, and kn, the slope factor, was -13.1 ± 0.3 mV. Error bars are s.e.m. and are not as large as the graph symbols.

Fig. 4C depicts the activation of elk1 and rat eag1 channels from different holding potentials. Holding potentials were varied over the range -120 mV to -40 mV in 20 mV increments. Test potential was 0 mV. The difference in time scale for the two types of channel is to be noted.

Fig. 4D shows the deactivation of elk1 channel tail currents. Currents were activated by a 2500 ms step to +10 mV followed by steps to potentials over the range -90 to -30 mV in 10 mV increments. Time constants for deactivation are indicated next to the appropriate trace. The trace at -70 mV is not shown due to the small amplitude of the tail current.

Fig. 4E shows the activation of the elk1 channel in control perfusion solution (pH = 7.7, left) and in perfusion solution with pH lowered to 6.6 (right). Recordings show current responses to voltage steps from a holding potential of -70 mV. Test potentials are indicated next to selected current traces.

Fig. 4F depicts conductance-voltage curves in

perfusion solutions with various pH values. Data points were fitted with the equation:

$$G = Gmax/(1 + exp((V-Vn)/kn))$$

where the midpoint for activation Vn = 9.6, 8.6, 15.7, 25.2, and 9.0 mV, the slope factor kn = -13.8, -13.7, -12.5, -12.3, -13.4 mV and the maximum conductance Gmax = 26.8, 26.3, 24.1, 21.8, 25.8 µS for pH = 7.7, 8.6, 7.1, 6.6, and wash (7.7) respectively.

Fig. 4G shows that E4031 does not block the elk1 channel. Voltage-clamp protocol is the same as in Fig. 4A.

Fig. 4H shows the elk1 channel is blocked by 1 mM Ba2+. Voltage-clamp protocol is the same as in Fig. 4A.

Fig. 5A and 5B show autoradiographs of RNase protection assays showing *EAG* potassium channel mRNA expression in sympathetic ganglia, brain and sciatic nerve determined by RNase protection analysis.

Fig. 5A shows samples tested: superior cervical ganglia (SCG), coeliac ganglia (CG), superior mesenteric ganglia (SMG) and brain. For the sympathetic ganglia, the sample always contained 5 µg of total RNA. A variable amount of total RNA was included in the brain samples as noted. In gel a) *elk1* mRNA was expressed in all three sympathetic ganglia. The brain sample contained 5 µg of total RNA. In gel b) *elk2* mRNA was not expressed in sympathetic ganglia but was moderately abundant in brain. The brain sample contained 2.5 µg of total RNA. In gel c) *eag*1 mRNA was expressed at equal levels in all three ganglia and was also expressed in brain. The brain sample contained 14 µg of total RNA. In gel d) *eag2* mRNA was barely detectable in the SCG but was expressed in the two prevertebral ganglia, the CG and SMG, as well as in brain. The brain sample contained 14 µg of total RNA.

Fig. 5B shows *elk*1 and *erg1* mRNA expression in sciatic nerve (SN). Superior cervical ganglia (SCG) RNA served as a gpositive control. All samples contained 5 µg of total RNA.

The cyclophilin gene (cyc) was used as an internal positive control in all experiments to check for sample loss and equal loading.

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48. U.S. Patent No. 5,670,335

49. U.S. Patent No. 5,776,734.

DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

The present invention provides for the first time three nucleic acids encoding respectively *elk*1, *elk*2 or *eag*2 identified and cloned from rat and human tissue. The polypeptides encoded by *elk*1, *elk*2 or *eag*2 are members of the

voltage-gated eag-like family of potassium channel units (elk). Members of this family are polypeptides that are alpha subunits of slowly activated outward rectifier potassium channels. Outward rectifier potassium channels have significant roles in maintaining the resting potential and in controlling excitability of a cell.

The invention also provides methods of screening for modulators, activators, and inhibitors of inward rectifier potassium channels that contain a subunit selected from the group consisting of elk1, elk2 and eag2. Such modulators of outward rectifier channel activity are useful for treatment of hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism, hyperparathyroidism, paacreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency.

Furthermore, the invention provides assays for elk1, elk2 or eag2 activity where for elk1, elk2 or eag2 acts as a direct or indirect reporter molecule. Such uses of for elk1, elk2 or eag2 as a reporter molecule in assay and detection systems have broad applications, e.g., elk1, elk2 or eag2 can be used as a reporter molecule to measure changes in potassium concentration, in vitro, in vivo, and ex vivo, membrane potential, current flow, ion flux, transcription, signal transduction, receptor-ligand interactions, second messenger concentrations. In one embodiment, elk1, elk2 or eag2 can be used as an indicator of current flow in a particular direction (outward potassium flow), and in another embodiment, elk1, elk2 or eag2 can be used as an indirect reporter via attachment to a second reporter molecule such as green fluorescent protein. The invention provides for methods of detecting elk1, elk2 or eag2 nucleic acid and protein

expression allows investigation of channel diversity provided by elk1, elk2 or eag2 and the regulation/modulation of heteromeric channel activity provided by elk1, elk2 or eag2 , as well as disease diagnosis for conditions such as hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism, hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency Finally, the invention provides for a method of screening for mutations of elk1, elk2 or eag2 genes or proteins. The invention includes, but is not limited to, methods of screening for mutations in elk1, elk2 or eag2 with the use of a computer. Similarly, the invention provides for methods of identifying the three-dimensional structure of elk1, elk2 or eag2 , as well as the resulting computer readable images or data that comprise the three dimensional structure of elk1, elk2 or eag2. Other methods for screening for mutations of *elk*1, *elk*2 or *eag*2 genes or proteins include high density oligonucleotide arrays, PCR, immunoassays and the like. Functionally, each of the three elk1, elk2 or eag2 polypeptide is an alpha subunit of an slowly activated outward rectifier potassium channel. Such outward rectifier channels are heteromeric or homomeric and contain typically contain four alpha subunits or monomers each with two transmembrane domains. Outward rectifier possium channels comprising elk1, elk2 or eag2 can be heteromeric and may contain one or more alpha subunits of elk1, elk2 or eag2 along with one or more other alpha subunits from the EAG-like family. Each of elk1, elk2 or eag2 can also comprise a homomeric slowly activated outward rectifier channel. The presence of elk1, elk2 or eag2 in an slowly activated outward rectifier potassium channel

modulates the activity of the heteromeric channel and thus enhances channel diversity.

Structurally, the nucleotide sequence of elk1 (SEQ ID No:1) encodes a polypeptide of approximately 1102 amino acids with a predicted molecular weight of approximately 123 kDa (SEQ ID No:2) and a predicted range of 100-150 kDa.

Specific regions of the elk1 nucleotide and amino acid sequence may be used to identify polymorphic variants, homologues, and alleles of elk1. This identification can be made in vitro, e.g., under stringent hybridization conditions and sequencing, or by using the sequence information in a computer system for comparison with other nucleotide sequences. Sequence comparison can be performed using any of the sequence comparison algorithms discussed below. Antibodies that bind specifically to elk1 can also be used to identify alleles and polymorphic variants.

Polymorphic variants and alleles of elk1 can be confirmed by coexpressing the putative elk1 polypeptide and examining whether it forms a heteromeric slow activated outward rectifier potassium channel, when co-expressed with another member of the EAG-like family such as elk2. This assay is used to demonstrate that a protein haying about 80% or greater, preferably 90% or greater amino acid identity to elk1 shares the same functional characteristics as elk1 and is therefore a species of elk1. Typically, elk1 having the amino acid sequence of SEQ ID No:2 is used as a positive control in comparison to the putative elk 1 protein to demonstrate the identification of a polymorphic variant or allele of elk1.

Similarly, polymorphic variants or alleles of elk2 or eag2 may be identified. For elk2, the partial amino acid sequence SEQ ID NO:3 may be used. For EAG2, the partial amino

acid sequence SEQ ID NO:4 may be employed.

elk1 nucleotide and amino acid sequence information may also be used to construct models of a heteromeric slowly activated outward rectifier potassium channels in a computer system. These models are subsequently used to identify compounds that can activate or inhibit heteromeric slowly activated outward rectifier potassium channels comprising elk1. Such compounds that modulate the activity of channels comprising elk1 can be used to investigate the role of elk1 in modulation of channel activity and in channel diversity.

The partial amino acid sequences for elk2 and eag2 may also be used in like manner.

The isolation of biologically active elk1, elk2 and eag2 for the first time provides a means for assaying for inhibitors and activators of heteromeric slowly activated outward rectifier potassium channels that comprise elk1, elk2 or eag2 subunits. Biologically active elk1, elk2 or eag2 is useful for testing modulators, inhibitors, and activators of slowly activated outward rectifier potassium channels comprising subunits of elk1, elk2 or eag2 and other eag-like members using in vivo and in vitro expression that measure, e.g., changes in voltage or current. Such activators and inhibitors identified using a slowly activated outward rectifier potassium channel comprising at least one elk1, elk2 or eag2 subunit can be used to further study slowly activated outward rectification, channel kinetics and conductance properties of heteromeric channels. Such activators and inhibitors are useful as pharmaceutical agents for treating disorders such as hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism,

hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency. Methods of detecting elk1, elk2 or eag2 and expression of channels comprising elk1, elk2 or eag2 are also useful for diagnostic applications. For example, chromosomal localization of elk1, elk2 or eag2 can be used to identify diseases caused by and associated with elk1, elk2 or eag2 . Methods of detecting elk1, elk2 or eag2 are also useful for examining the role of elk1, elk2 or eag2 in channel diversity and modulation.ef channel activity.

Definitions

As used herein, the following terms have the meanings ascribed to them unless :specified otherwise.

The phrase "outward rectifier" activity or "outward rectification" refers to a characteristic of a potassium-channel allows potassium outflux, with little potassium influx. For example, potassium ions flow outward through such channels when the membrane potential is negative to EK, while at more positive membrane potentials, inward currents may be diminished (see, e.g., Pessia et al., EMBO J. 15:2980-2987 (1996)) EK is the membrane potential for potassium, the combination of the voltage potential and $[K^+]$ potential at which there is no net flow of potassium ion. This value (-98 mV) is also known as the "reversal potential" or the "Nernst" potential for potassium. Typically, the channel is composed of four subunits and the channel can be heteromeric or homomeric. The characteristic of outward rectification can be measured by a variety of techniques for measuring changes in current flow and ion flux through a channel, e.g., by changing the [K+] of the external solution and measuring the activation potential of the channel current (see, e.g., U.S. Patent No. 5,670,335), by measuring current with patch clamp techniques

under different conditions, and by measuring ion flux with radiolabeled tracers or voltage-sensitive dyes under different conditions.

A "beta subunit" is a polypeptide monomer that is an auxiliary subunit of a cation-chanel composed of alpha subunits; however, beta subunits alone cannot form a channel (see, e.g., U.S. Patent No. 5,776,734). Beta subunits are known, for example, to increase the number of channels by helping the alpha subunits reach the cell surface, change activation kinetics, and change the sensitivity of natural ligands binding to the channels. Beta subunits can be outside of the pore region and associated with alpha subunits comprising the pore region. They can also contribute to the external mouth of the pore region.

"elk1" refers to a polypeptide that is a subunit of an slowly activated outward rectifier potassium channel and a member of the EAG family of potassium channels. "elk1" therefore refers to polymorphic variants, alleles, mutants, and closely related interspecies variants that: (1) have amino acid sequence identity of at least 60% , preferably about 80-90%, to elk1; or (2) have a nucleic acid sequence that specifically hybridize under stringent hybridization conditions to a sequence of about 17-25 base pairs selected from SEQ ID No:1 or a conservatively modified variant thereof; or (4) are amplified by primers that specifically hybridize under stringent hybridization conditions to the same sequence as a primer set consisting of SEQ ID No:5 and SEQ ID No:6.

"elk2" therefore refers to polymorphic variants, alleles, mutants, and closely related interspecies variants that: (1) have amino acid sequence identity of at least 60% , preferably about 80-90%, to elk2; or (2) have a nucleic acid

sequence that specifically hybridize under stringent hybridization conditions to a sequence of about 17-25 base pairs selected from from SEQ ID No:13 or a conservatively modified variant thereof; or (4) are amplified by primers that specifically hybridize under stringent hybridization conditions to the same sequence as a primer set consisting of SEQ ID No:9 and SEQ ID No:10.

"eag2" therefore refers to polymorphic variants, alleles, mutants, and closely related interspecies variants that: (1) have amino acid sequence identity of 50-90% , preferably about 80-90%, to eag2; or (2) have a nucleic acid sequence that specifically hybridize under stringent hybridization conditions to a sequence of about 17-25 base pairs selected from sequence SEQ ID No:14 or a conservatively modified variant thereof; or (4) are amplified by primers that specifically hybridize under stringent hybridization conditions to the same sequence as a primer set consisting of SEQ ID No:5 and SEQ ID No:6.

The phrase "functional effects" in the context of assays for testing compounds that may affect "elk1", "elk2" or "eag2" channels includes the determination of any parameter that is indirectly or directly under the influence of the "elk1", "elk2" or "eag2" channel. It includes changes in ion flux, membrane potential, current flow, transcription, G-protein binding, phosphorylation or de-phosphorylation, signal transduction, receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP3, or intracellular Ca2+), as measured in vitro, in vivo, and ex vivo, and also includes other physiologic effects such increases or decreases of neurotransmitter or hormone release.

By "determining the functional effect" is meant assays

for a compound that increases or decreases a parameter that is indirectly or directly under the influence of "elk1", "elk2" or "eag2". Such functional effects can be measured by any means known to those skilled in the art, e.g., patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux, inducible markers, oocyte or tissue culture cell expression of elk1, elk2 or eag2; transcriptional activation; ligand binding assays; voltage, membrane potential and conductance changes; ion flux assays; changes in intracellular second messengers such as cAMP and inositol triphosphate (IP3); changes in intracellular calcium levels; neurotransmitter release, and the like.

"Inhibitors," "activators," and "modulators" of the eag-like slowly activated outward rectifying potassium channels comprising elk1, elk2 or eag2 refer to modulator molecules identified using in vitro and in vivo assays for channel function. Inhibitors are compounds that decrease, block, prevent, delay activation, inactivate, desensitize, or down regulate the channel. Activators are compounds that increase, open, activate, facilitate, enhance activation, sensitize or up regulate channel activity. Modulators include naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like. Assays for inhibitors and activators include, e.g., coexpressing a elk1 subunit with another eag-like alpha subunit in cells or cell membranes, applying putative modulator compounds, and then measuring flux of ions through the eag-like channel and determining the functional effect of the modulator as described above.

"Biologically active" "elk1", "elk2" or "eag2" refers to "elk1", "elk2" or "eag2" that comprises a potassium channel having slowly activated outward rectifier activity tested as

described above. Typically, the potassium channel is heteromeric and contains at least one, preferably two subunits of elk1 or elk2 or eag2 and subunits of another eag-like family member such as eag1.

The terms "isolated" "purified" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated *elk*1 nucleic acid is separated from open reading frames that flank the *elk*1 gene and encode proteins other than *elk*1. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably 99% pure.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Polypeptides can be modified, e.g., by the addition of carbohydrate residues to form glycoproteins. The terms "polypeptide," "peptide" and "protein" include glycoproteins, as well as nonglycoproteins.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the generic code. Synthetic amino acids include naturally occurring amino acids that are modified, e.g., hydroxyproline, carboxyglutamate, and o-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group., e.g., homoserine, norTeucine, methionine sulfoxide, and methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the

general chemical structure of an amino acid, but that functions in a manner similar o a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides may be referred to by their commonly acceptd single-letter codes (A, T, G, C, U, etc.).

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Obtsuka et al., J. Biol. Chem. 26O:26OS-26O8 (1985); Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)). Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon in an amino acid herein, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of

skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphc variants and alleles of the invention.

The following groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);
2) Serine (S), Threonine (T);
3) Aspartic acid (D), Glutamic acid (E);
4) Asparagine (N), Glutamine (Q);
5) Cysteine (C), Methionine (M);
6) Arginine (R), Lysine (K), Histidine (H);
7) Isoleucine (I), Leucine (L), Valine (V); and
8) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(see, e.g., Creighton, Proteins (1984) for a discussion of amino acid properties). .

Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, see, e g., Alberts et al., Molecular Biology of the Cell (3rd ed., (1994) and Cantor & Schimmel, Biophysical Chemistry Part I: The Conformation of Biological Macromolecules (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 50 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of ß-sheet and α-helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the non-covalent association of independent tertiary units

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (e.g., the polypeptide of SEQ ID No:1 can be made detectable, e.g., by incorporating a radiolabel into the peptide, and used to detect antibodies specifically reactive with the peptide).

As used herein a "nucleic acid probe or oligonucleotide" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through

complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarily with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

A "labeled nucleic acid probe or oligonucleotide" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non recombinant) form of the cell or

express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g;, a, fusion protein).

An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences (i.e., a human *elk*1 monomer tail region) that are the same or have a specified percentage of amino acid residues or nucleotides (i.e., 80% identity, preferably 85%, 90%, or 95% identity) that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences are then said to be "substantially identical." This definition also refers to the complement of a test sequence. Preferably, the percent identity exists over a region of the sequence that is at least about 25 amino acids in length, more preferably over a region that is 50 or 100 amino acids in length.

For sequence comparison, one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, as described herein, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program

parameters.

A "comparison window," as used herein, includes reference to a segment of contiguous positions having a length of at least about 25 nucleotides, usually about 50 to about 200 nucleotides, more usually about 100 to about 150 nucleotides in which a sequence may be compared to a reference sequence after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat '1. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, S75 Science Dr., Madison, WI), or by. manual alignment and visual inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:35 1-360 (1987). The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino-acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most

related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g., version 7.0 (Devereaux et al., Nucleic Acids Res. 12:387-39S (1984)).

Another example of algorithm that is suitable for determining percent .sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncti.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short-words of length W in the query sequence, which either match or satisfy some positive valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment

score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Nat 'l. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two

molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleotide sequences are substantially identical is that the same primers can be used to amplify both sequences.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or

other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5X SSC, and 1% SDS, incubating at 42°C, or, 5X SSC, 1% SDS, incubating at 65°C, with a wash in 0.2X SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at.37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

"Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classifled as either kappa or lambda. Heavy

chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, lgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetrameter is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 5O-70 kDa). The N-terminus of each chain defines a variable region of about 1OO to 11O or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively.

Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)': a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'2 dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (see Fundamental Immunology (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty et al., Nature 348:552554 (199O)).

An "anti-elk1 " antibody is an antibody or antibody fragment that specifically binds a polypeptide encoded by the *elk*1 gene, cDNA, or a subsequence thereof. Similarly, an anti-elk2 antibody is an antibody or antibody fragment that specifically binds a polypeptide encoded by the *elk*2 gene, cDNA, or a subsequence thereof and an anti-eag2 antibody is an antibody or antibody fragment that specifically binds a polypeptide encoded by the *eag*2 gene, cDNA, or a subsequence thereof .

A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

The term "immunoassay" is an assay that uses an antibody to specifically bind an antigen. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the

specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity to a particular protein. For example, polyclonal antibodies raised to rat *elk*1 with the amino acid sequence of the tail region encoded in SEQ ID No:2 can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with the tail region of rat elk1 and not with other proteins, except for polymorphic variants and alleles of rat elk1. This selection may be achieved by subtracting out antibodies that cross react with molecules such as human elk1 and other elk molecules such as elk2. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

The phrase "selectively associates with" refers to the ability of a nucleic acid to "selectively hybridize" with another as defined above, or the ability of an antibody to selectively (or specifically) bind to a protein, as defined above.

By "host cell" is meant a cell that contains an expression vector and supports the replication or expression of the expression vector. Host cells may be prokaryotic cells

such as E. coli, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells such as CHo, HeLa and the like.

"Biological sample" as used herein is a sample of biological tissue or fluid that contains *elk*1 or nucleic acid encoding elk1 protein. Such samples include, but are not limited to, tissue isolated from humans. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. A biological sample is typically obtained from a eukaryotic organism, preferably eukaryotes such as fungi, plants, insects, protozoa, birds, fish, reptiles, and preferably a mammal such as rat, mice, cow, dog, guinea pig, or rabbit, and most preferably a primate such as chimpanzees or humans.

Isolation of cDNA clones

According to the present invention, three rat *eag*-like genes, *elk*1 and *elk2*, and *eag2 were* isolated by PCR amplification of cDNAs from rat brain and SCG cDNA. For *elk*1 degenerate primers directed against the S1 and S5 regions were used. For *elk2*, the *elk1* sequence was used to identify similar sequences in a GenBank search. Two overlappiing expressed sequence tags were found to encode a closely related channel (GenBank accession numbers R35526 and R73353). These fragments encompassed part of the cytoplasmic amino terminal domain through to the S2 region. The complementary oligonucleotide sequences derived from these sequences were used to amplify cDNA clones from rat brain and SCG cDNA.

Based *on* the sequences of the cDNAs obtained, the deduced amino acid sequence of *elk*1 (SEQ ID NO:2) and a partial amino acid sequence of *elk2* (SEQ ID NO:3) were determined.

Using the same primers for isolating the partial *elk*1

cDNA, the *eag*2 gene was initially identified by PCR amplification from rat brain cDNA and a partial amino acid sequence of *eag*2 (SEQ ID NO:4) was determined.

RNA was prepared from sympathetic ganglia and brain using the *RNase* protection assay. Full length *elk*1 and *eag*1 cRNA transcripts were synthesized *in vitro*. The full length rat *elk*1 cDNA was isolated as described. The full length rat *eag*1 cDNA was amplified from rat SCG cDNA using the Expand High Fidelity PCR system. Expression was conducted in *Xenopus* oocytes. The details of the procedures used are described hereinbelow.

The human genes for *elk*1, *elk*2 and *eag*2 may be obtained by searching the GenBank EST database using the corresponding rat gene sequences, SEQ ID NO:1, SEQ ID NO:13 or SEQ ID NO:14, either with the BLAST or PILEUP programs. The search identifies EST from human tissues that are homologous with the rat *elk*1, *elk*2 or *eag*2 gene. The region having a sequence that is the most homologous with the rat *elk*1 *elk*2 or *eag*2 gene is analyzed to select three sets of primers. In accordance with the procedures described herein below, one of the three primers is used to synthesize cDNA respectively for human *elk*1, *elk*2 and *eag*2 gene from homogenized human fetal brain, human adrenal and human stellate ganglia mRNA. The entire open reading frame of the human *elk*1 gene is then determined using RACE PCR (Frohman, 1994) using the other two EST specific primers as the initial anchor oliogonucleotides. Several rounds RACE may be required to obtain the complete open reading frame of the human *elk*1, *elk*2 and *eag*2 respectively.

A systematic search for new members of the *EAG* gene family was conducted using two different methods: i) novel

genes were isolated using degenerate primers combined with PCR amplification of cDNAs, ii) novel genes were identified in searches of GenBank and cDNAs then isolated by PCR. Three new genes were identified.

elk1

The *elk1* gene was initially identified using the following degenerate primers directed against the S1 and S5 regions to PCR amplify partial cDNA clones from rat brain and SCG cDNA.

Forward: TTYAARRCNRYNTGGGAYTGG (SEQ ID NO:5)

Reverse: RTACCADATRCANGCNAGCCARTG (SEQ ID NO:6)

An initial sequence encompassing the entire open reading frame of the *elk1* gene was determined by performing 5′ and 3′ RACE PCR (Frohman, 1994) using initial anchor oligonucleotides complementary to the partial *elk1* cDNA clone. Obtaining a sequence encompassing a complete open reading frame required several rounds of RACE in both directions using SCG cDNA as a template for amplification.

Once cDNAs were obtained that extended beyond both the 5′ and 3′ ends of the open reading frame, oligonucleotides complementary to non-coding regions at either end of the coding sequence were designed. Multiple full-length cDNA clones were amplified in independent PCR reactions from rat SCG cDNA using the Expand High Fidelity PCR system (Boehringer-Mannheim, Indianapolis, IN). The following oligonucleotides were used to amplify full-length cDNA clones, giving a 52 b.p. and 381 b.p. 5′ and 3′ UTR respectively.

forward: CGGGATCCTTGTGGACAAAC (SEQ ID NO:7)

reverse: TTCAGGAATGACAACCAGGC (SEQ ID NO:8)

Two independent clones were sequenced, in their entirety, using a combination of manual and automatic

sequencing. Differences between the two sequences were resolved by partial sequencing of a third independent full-length cDNA clone. Sequence alignment of the deduced amino acid sequence was performed using the ClustalW program (Thompson et al, Nucleic Acids Res. (1994) 22:673-4680. The cDNA sequence of the *elk1* gene is shown in Fig. 3.

elk2

The rat *elk2* gene was initially identified by searching GenBank using the elk1 sequence. Two overlapping expressed sequence tags were found to encode a closely related channel (GenBank accession numbers R35526 and R73353). These fragments encompassed part of the cytoplasmic amino terminal domain through to the S2 region. The following complementary oligonucleotide sequences derived from these sequences were used to amplify cDNA clones from rat brain and SCG cDNA.

forward: GTGATACCCATAAAGAATGAG (SEQ ID NO:9)

reverse: CGGAAATTCAGCACAATGTC (SEQ ID NO:10)

The *elk2* sequence is

AAGGGGGAGG TGGCCCTCTT CCTGGTCTCT CACAAGGACA TCAGTGAGAC
CAAGAACCGA GGAGGCCCTG ACAACTGGAA GGAGAGAGGT GGTGGCCGAC
GCAGATATGG TCGGGCAGGA TCCAAAGGCT TTAATGCCAA TCGGAGGCGC
AGCCGGGCGG TTCTCTACCA CCTCTCTGGT CACCTGCAGA AACAACCCAA
GGGCAAGCAC AAACTCAATA AGGGTGTGTT TGGAGAGAAG CCAAATTTGC
CCGAATATAA AGTCGCTGCT ATCCGGAAGT CACCCTTTAT CCTGCTGCAC
TGTGGGGCTC TGAGAGCCAC CTGGGATGGC TTCATCCTGC TCGCCACGCT
CTACGTGGCT GTCACTGTGC CATACAGCGT GTGTGTGAGC ACAGCACGGG
AGCCCAGTGC TGCCCGTGGC CCACCTAGTG TCTGTGACCT GGCCGTGGAA
GTCCTCTTCA TCTT (SEQ ID NO:13)

The deduced amino acid sequence of elk2 is:

KGEVALFLVS HKDISETKNR GGPDNWKERG GGRRRYGRAG SKGFNANRRR
SRAVLYHLSG HLQKQPKGKH KLNKGVFGEK PNLPEYKVAA IRKSPFILLH
CGALRATWDG FILLATLYVA VTVPYSVCVS TAREPSAARG PPSVCDLAVE
VLFI (SEQ ID NO:3)

The *elk2* gene was clearly a member of a subfamily of mammalian elk genes. The *elk2* deduced amino acid sequence was 52% identical to *elk1* and only 27% and 29% identical to rat *erg1* and *EAG1* respectively over the region shown above.

The rat *elk2* gene was also used to search the GenBank human EST to obtain the human *elk2* gene, and elk2 polypeptide.

eag2

The *eag2* gene was initially identified by PCR amplification from rat brain cDNA using the same primers as were used to isolate the partial rat elk1 cDNA. The reported sequence encompasses a region between S1 and S5.

The *eag2* sequence is:

GTGATTTTAA TTCTTACCTT CTACACCGCC ATCATGGTTC CTTACAACGT
TTCCTTCAAA ACAAAACAGA ACAATATCGC CTGGCTGGTT CTGGACAGCG
TGGTGGACGT TATTTTTCTG GTGGACATCG TTTTAAACTT TCACACGACT
TTTGTGGGGC CGGGTGGAGA GGTCATTTCT GACCCAAAAC TCATACGGAT
GAACTATCTG AAAACTTGGT TTGTGATTGA TCTGCTGTCT TGTTTACCTT
ATGACATCAT CAATGCCTTT GAAAATGTGG ATGAGGGAAT CAGCAGTCTC
TTCAGCTCTT TAAAGGTGGT ACGCCTCTTA CGCCTGGGCC GTGTTGCTAG
GAAACTGGAC CATTACCTGG AATATGGAGC AGCGGTCCTT GTGCTCCTGG
TATGTGTGTT TGGACTGGTT GCC (SEQ ID NO:14)

The deduced amino acid sequence of eag2 is:

VILILTFYTA IMVPYNVSFK TKQNNIAWLV LDSVVDVIFL VDIVLNFHTT
FVGPGGEVIS DPKLIRMNYL KTWFVIDLLS CLPYDIINAF ENVDEGISSL
FSSLKVVRLL RLGRVARKLD HYLEYGAAVL VLLVCVFGLV A
(SEQ ID NO:4)

The *eag*2 gene was very similar to the previously identified rat *eag* gene (Ludwig et al., 1994), being 72% identical to rat *eag* (*eag*1) at the DNA sequence level over this region. The deduced amino acid sequence of eag2 was 92% identical to eag1 over this region and only 42% and 45% identical to rat elk1 and erg1 respectively.

RNase Protection Assay

The procedures for the preparation of RNA from sympathetic ganglia and brain and the performance of the RNase protection assay were identical to those described previously. Dixon and McKinnon, Eur. J. Neurosci. (1996) 8:83-191.

All RNA samples were prepared from pooled dissections of sympathetic ganglia from young adult Sprague-Dawley rats (4-5 weeks). The superior cervical, coeliac, and superior mesenteric ganglia were used because they are relatively discrete and can be routinely identified for dissection. For RNA preparation three tissue samples were used: (i) the combined left and tight SCG, (ii) the right CG and (iii) the right SCG. Ganglia were carefully dissected free of host tissue, rinsed in phosphate buffered saline and then frozen in liquid nitrogen. Frozen ganglia were then homogenized in guanidinium thiocyanate and total RNA was prepared by pelleting over a CsCl step gradient. All RNA smaples were carefully quantitated by spectrophotometic analysis and the integrity of the RNA samples was confirmed by analysis on a denaturing agarose gel. Sciatic nerve RNA was prepared similarly following dissection of sciatic nerve from rat hind limb and rapid freezing in liquid N_2. RNA expression was quantitated directly from dried RNase protection gels using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The RNase protection assay was performed as follows.

RNA samples were assayed as a set coming from the same group of animals. The data were obtained from independent sets of RNA samples. For each sample point 5 or 10 μg of total RNA was used in the assay. For the positive control brain or atria sample, 5 μg of total RNA was used. In experiments where the aim was to compare the level of expression of two different K channels, the RNA probes were made using two DNA templates in the same reaction mixture to minimize differences in the specific activity of the two probes, For comparisons between probes, the data were corrected for differences in specific activity of the probes by assuming that the specific activity was directly proportional to the number of UTPs in each sequence. A probe for the rat cyclophilin gene was included in the hybridization as an internal control. The cyclophilin probe had a specific activity approximately sevenfold lower than that of the K channel probes. 10 μg of yeast tRNA was used as a negative control to test for the presence of probe self-protection bands. RNA expression was quantitated directly from dried Rnase protection gels that were placed in a β-counter (Betascope 603, Biogen). Specific counts for each band were determined by subtracting a background signal determined from the tRNA lane using a counting rectangle of the same size and relative location as was used for the sample band.

The following DNA templates were used for the RNase protection assays.

*eag*1: Encompasses nucleotides 855 to 1162 of rat *eag* (Ludwig et al, EMBO J. (1994) 13:451-4458. GenBank Accession number Z34264).

*eag*2: Used partial *eag*2 cDNA described above (GenBank Accession number: AF073891).

*elk*1: Encompasses nucleotides 710 to 1177 of rat *elk*1 described above (GenBank Accession number: AF061957).

*elk*2: Used partial *elk*2 cDNA described above (GenBank Accession number: AF073892).

Expression in *Xenopus* Oocytes

Full-length rat *elk*1 and *eag*1 cRNA transcripts were synthesized in vitro. Isolation of the full-length rat *elk*1 cDNA is described above. The full-length rat *eag*1 cDNA was amplified from rat SCG cDNA using the Expand High Fidelity PCR system (Boehringer-Mannheim, Indianapolis, IN). The following oligonucleotides were used to amplify full-length EAG1 cDNA clones:

forward : TGCTGCGGTGAGACACG (SEQ ID NO:11)

reverse : TGGTCATGTGTTTGGTGCG (SEQ ID NO:12)

Oocytes were prepared from mature female *Xenopus laevis* using established procedures (Colman, 1984, incorporated herein by reference). Frogs were anaesthetized in ice water containing a 0.1% solution of Tricaine. Defolliculation was performed by incubation for 2 hours in 2 mg/ml collagenase (Type VIII, Sigma, St. Louis, MO) in Ca^{2+}-free OR2 oocyte medium with gentle agitation. Oocytes were stored in OR3 solution (50% L-15 medium (GIBCO, Gaithersburg, MD), 1 mM glutamine, 15 mM Na-HEPES (pH 7.6), 0.1 mg/ml gentamicin) at 18°C. Oocytes were injected with 50 nl of cRNA (~0.3 ng/nl) using a microdispenser and a micropipette with tip diameter of 10-20 μm. Injected oocytes were incubated at 18°C for 24-48 hours prior to analysis.

Oocytes were voltage-clamped using a two-microelectrode voltage clamp. Intracellular electrodes filled with 3 M KCl with resistances of 0.5-3 MΩ were used. The

standard extracellular recording solution contained: 80 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Na-HEPES (pH 7.6). Data collection and analysis were performed using pClamp software (Axon Instruments, Foster City, CA). E4031 was obtained from Eisai Co. Ltd. (Tokodai, Japan).

Human *elk1*

Two methods described below are useful for obtaining the cDNAs that extend beyond the 5' and 3' ends of the open reading frame of the human *elk1* gene.

Method 1

The DNA sequence for the rat *elk1* gene (SEQ ID NO:1) is used to search the GenBank data base in order to identify expressed sequence tags (EST) from human tissues that are homologous with a part of the gene. The ESTs are not be known to encode any part of the human *elk1* gene.

Using the *elk1* gene (SEQ ID NO:1) and applying the BLAST program, a human EST with the highest degree of homology to the *elk1* gene is identified.

Three primers of 17-25 base pairs having at least 90% homology with a region of rat *elk1* are selected from the EST sequence identified. The primer from the most 3' end of the EST is used to make cDNA from RNA isolated from human tissue. The tissue sources include, but are not restricted to, human brain, human adrenal and human stellate ganglia. cDNA synthesis is performed using established techniques as described by the manufacturers of the reverse transcriptase enzyme. The temperature recommended by the manufacturer and , a higher temperature (50°C) are used to synthesize the cDNA. The higher temperature of 50°C is used to maximize the specificity of the reaction for the human *elk1* gene.

The entire opening reading frame of the human elk1 gene is obtained using 5' RACE PCR (Frohman, 1994) using the two other primers specific for the EST as the initial anchor oligonucleotides. The procedure is described for the rat *elk*1 gene herein above. Obtaining a sequence encompassing a complete open reading frame may require several rounds of RACE.

The procedures for the preparation of RNA and the expression of the cDNA transcripts in *Xenopus Oocytes* are as described above for rat *elk*1.

Method 2

Degenerate oligonucleotides are used to clone a partial cDNA sequence using human brain, adrenal or stellate cDNA as a template for amplification. Initially, the primers selected as described above are directed against the core S1 to S6 region of the gene. However, other regions may also be used.

The primers selected include codons for amino acids that are specific for the *elk*1 gene and not found in related genes.

The entire opening reading frame of the human *elk*1 gene is determined using 5' and 3' RACE PCR (Frohman, 1994) using initial anchor oligonucleotides complementary to the partial human elk1 cDNA clone.

Obtaining a sequence encompassing a complete open reading frame may require several rounds of RACE in both directions.

Once cDNAs are obtained that extend beyond both the 5' and 3' ends of the open reading frame, oligonucleotides are selected complementary to non-coding regions at either end of

the coding sequence. Multiple full-length cDNA clones are amplified in independent PCR reactions from human brain, adrenal or stellate cDNA using the standard amplification techniques as described the manufacturer of the DNA polymerase.

Independent clones are sequenced, in their entirety, using automatic sequencing. Sequence differences are resolved by partial sequencing of an independent cDNA clone. Sequence alignment of the deduced amino acid sequence are performed using the ClustalW program (Thompson et al., 1994).

RESULTS

Isolation of Full-length *elk*1 cDNA Clone

A systematic search for genes related to the *Drosophila elk* gene and the mammalian *eag* gene was conducted and three new genes are identified in rat: *elk*1, *elk*2 and *eag*2 and in human (see Methods). Of these three genes the *elk*1 gene is of the most interest since no *elk* channel had been expressed and characterized previously. In addition the rat *elk*1 gene is expressed in sympathetic ganglia, a system that has been well characterized both in terms of potassium channel function as well as gene expression (Dixon and McKinnon, 1996, supra; Shi *et al.*, 1997, supra).

The deduced amino acid sequence of the *elk*1 gene suggests that it encodes a protein 1,102 amino acid residues in length with an estimated molecular weight of 123 kDa (Fig. 1). Assignment of the initiator methionine and the start of the open reading frame was facilitated by the high degree of similarity in this region between the *elk*1 and *Drosophila elk* sequences. There were multiple stop codons in all three reading frames around the selected stop codon.

The deduced elk1 amino acid sequence was 41% identical to the *Drosophila elk* sequence. Identity with other members of the *EAG* gene family was significantly lower, ranging from 26 to 31%. This result suggests that the *elk1* gene is a mammalian equivalent of the *Drosophila elk* gene. A similar level of identity between mammalian *erg* genes and their *Drosophila* counterpart has been found previously to be in the range 41 to 44%. Shi et al, 1997, supra. Identity between the rat *eag*1 gene and the *Drosophila eag* gene is somewhat higher, however, at 51%.

There is a high degree of similarity between the *elk*1 deduced amino acid sequence and the *erg* and *EAG* sequences in the amino terminus, the hydrophobic core and the putative cyclic nucleotide binding domain (Fig. 1). The *elk*1 sequence shares the pore signature sequence GFG with both the *erg* and EAG channels, in contrast to most other potassium channels, which have GYG in the equivalent position (Wei et al, 1996, supra). The *elk*1 protein has a relatively large cytoplasmic carboxyl domain compared to the other two channels and there is a distinctive run of negatively charged residues in this region, with 11 of 12 residues being either glutamate or aspartate. A similar but much shorter sequence is seen in *EAG*1 in the equivalent position.

Functional Expression of *elk*1 channels

It was of interest to compare the biophysical properties of the elk1 channel with those of eag and erg channels, since all three channels share significant regions of sequence identity. eag and erg channels have one common feature, they are relatively slowly activating compared to Kv-class (*Shaker*-related) channels. This property is also shared

by the elk1 channel (Fig. 4A). The elk1 channel is one of the slowest activating EAG channels, having a time constant for activation of 676 ± 37 ms (n = 7) at 0 mV. The threshold for activation is around -40 mV and the conductance-voltage curve can be fit with a simple Boltzmann distribution (Fig 4B: V_n = 9.3 ± 0.4 mV and k_n = -13.1 ± 0.3 mV (n = 7)).

In a simple activation protocol such as the one shown in Figure 3A, the elk1 channel has a similar waveform to eag channels: it is a slowly activating outward rectifier. he elk1 channel exhibits no apparent inactivation during depolarizing voltage steps of up to several seconds, similar to the mammalian eag1 channel (Ludwig et al, 1994, supra; Robertson et al, 1996, supra). In contrast, erg channels have very fast inactivation rates, significantly faster than their activation rates, which results in an apparent inward rectification at positive membrane potentials (Sanguinetti et al, 1995, supra; Spector et al, J. Gen. Physiol. (1996) 107:11-619).

One particularly striking feature of the eag channels is that the rate of activation is strongly dependent upon the holding potential, activation is significantly faster from depolarized than hyperpolarized holding potentials. This suggests that during the activation process the eag channel undergoes a slow, voltage-dependent transition between at least two distinct closed states before reaching the open state (Ludwig et al., 1994, supra; Robertson et al., 1996, supra). The elk1 channel shows no obvious change in the activation rate from different holding potentials, in marked contrast to the results for eag channels over a similar range of holding potentials (Fig. 4C). In this respect, the elk1 channel is more similar to *erg* channels, which also do not exhibit a significant shift in activation rates with changes

in holding potential.

There are some other kinetic properties for which the elk1 channel is more similar to erg than eag channels. The rate of deactivation of the elk1 channel is relatively slow, with a time constant of 111 ± 7 ms (n = 7) at -50 mV (Fig 4D). Deactivation rates for erg channels are also very slow, with deactivation time constants in the range of 100 ms up to several seconds at -50 mV (Sanguinetti et al, 1995, supra). In marked contrast, the deactivation rate of the mammalian eag1 channel is very fast, with a time constant of 1-6 ms (Terlau et al, Pflugers Arch. (1996) 432:301-312).

Activation of the elk1 channel was strongly dependent on the pH of the external bath solution (Fig. 4E). Following acidification of the external perfusate solution, the conductance-voltage curve was shifted significantly to more positive membrane potentials (Fig. 4F). The midpoint of the activation curve was shifted from 10.4 ± 0.4 mV at pH = 7.7, to 17.0 ± 0.7 mV at pH = 7.1 ($p < 0.05$), to 28.1 ± 1.5 mV at pH = 6.6 ($p < 0.001$). The slope factor was not significantly affected by changes in external pH ($p > 0.5$). Changing the external solution to a more alkaline pH did not produce significant shifts in the activation-conductance curve (Fig. 4F). A somewhat similar dependence of activation on external pH is seen for mammalian eag channels (Terlau et al, 1996, supra), although for eag channels there is also significant slowing of the activation kinetics by acidic pH, which is not seen for elk1 channels.

The elk1 channel was almost completely resistant to the application of two common potassium channel blockers, TEA and 4-AP. Concentrations of up to 10 mM of either drug had limited or no effects on the channel. Another class of

potassium channel blockers, methanesulfonanilide drugs such as E4031, are useful rEAGents because they are apparently quite selective for cloned and native *erg* channels at concentrations less than ~10 μM (Sanguinetti and Jurkiewicz, J. Gen. Physiol. (1990) 96:95-215). These compounds have not been tested systematically on closely related channels, however, and for this reason we examined the effects of E4031 on the elk1 channel. E4031 had almost no effect at concentrations up to 10 μM (Fig. 4G). Barium ions also have some potential for differentiating among the various *EAG*-family channels. The elk1 channel is more sensitive than the *EAG*1 channel to Ba^{2+} ions, being 85% blocked by 1 mM Ba^{2+} in the external solution (Fig. 4H). In comparison, the *EAG*1 channel is blocked less than 40% by 1 mM Ba^{2+} (see also Terlau et al., 1996, supra).

eag Gene Expression in Sympathetic Ganglia

To date the functional roles of eag, erg and elk channels have been poorly characterized *in vivo*. One potentially useful model system to study the function of these channels is the peripheral sympathetic ganglia. For this reason, we determined the distribution of *elk* and *eag* gene expression in three sympathetic ganglia: one paravertebral ganglia, the superior cervical ganglia (SCG) and two prevertebral ganglia, the coeliac ganglia (CG) and the superior mesenteric ganglia (SMG) (Fig. 5A). The *elk*1 gene is expressed in both prevertebral and paravertebral ganglia at approximately equal levels. In contrast, the *elk*2 gene is not expressed at detectable levels in sympathetic ganglia, although it is robustly expressed in the brain. Both *eag* genes are expressed in sympathetic ganglia. The *eag*1 gene is expressed at high levels in all three sympathetic ganglia. The *eag*2 gene is only expressed at significant levels in the prevertebral ganglia, the CG and SMG.

In addition to neurons, sympathetic ganglia also contain glial cells (known as satellite cells and developmentally related to Schwann cells). To determine whether gene expression in ganglia could be due to expression in glial cells we examined *eag* gene expression in sciatic nerve, which contains a relatively pure population of Schwann cells. Two of the seven *eag* genes tested, *elk*1 and *erg*1 are expressed in sciatic nerve (Fig. 5B).

A summary of *EAG* gene expression in sympathetic ganglia and sciatic nerve is shown in Table 1. At least six *EAG* genes are expressed in the sympathetic ganglia and all seven EAG genes are expressed in at least one neuronal tissue. Two genes, *eag*2 and *erg*2, are differentially expressed in sympathetic ganglia. Both genes are expressed at higher levels in prevertebral ganglia than paravertebral ganglia. The housekeeping gene cyclophilin was expressed at significantly lower levels in sciatic nerve RNA than in ganglia or brain RNA (Fig. 5B). It is possible, therefore, that mRNA levels, as a fraction of total RNA, are significantly lower in sciatic nerve than in ganglia or brain. For this reason, the relative level of *elk*1 and *erg*1 expression in sciatic nerve described in Table 1 may be an underestimate in comparisons between sciatic nerve and ganglia or brain.

It has been found in contrast to the *erg* genes, the *elk* genes are not expressed in heart tissue. This characteristic combined with the difference in electrophysiological characteristics should provide a tool for identifying pharmaceuticals which would block the K channels differentially. In this manner, K channel blockers which would intervene in arrythmia without interfering with the sympathetic nervous system may be identified.

Table 1

Distribution of EAG Potassium Channel Gene Expression in Rat Sympathetic Ganglia, Brain and Sciatic Nerve

	SCG	CG	SMG	Brain	Sciatic Nerve
eag1	+++	+++	+++	++++	-
eag2	+/-	+	+	++	-
erg1	+++++	+++++	+++++	+++++	+++
erg2	+	++	+++	-	-
erg3	+++	+++	+++	++++	-
elk1	+++	+++	+++	+/-	++
elk2	-	-	-	+++	-

Key:
+++++ very high, ++++ high, +++ moderate, ++ low, + very low,
+/- just detectable, - negative

Semi-quantitative analysis of the relative levels of expression of all the EAG genes was achieved by a combination of radioactive counting of individual gels and visual comparison of autoradiographs from multiple experiments. In every case the data are based on multiple independent experiments (n = 2 to 6). Data for sciatic nerve may be subject to a systematic underestimate (see Results). Data for the *erg* gene expression in sympathetic ganglia is adapted from Shi *et al.* (1997).

Discussion

The complete coding sequence of a new potassium channel gene, the rat *elk*1 gene, and partial sequences for two other *eag* genes: *eag*2 and *elk*2 and the procedures for obtaining the human *elk*1, *eag*2 and *elk*2 have been provided. The *elk*1 and *elk*2 genes are mammalian equivalents of the *Drosophila elk* gene. When expressed in a heterologous expression system, the elk1 channel has some similar biophysical properties compared to the related eag and erg channels, but also has some clearly distinct properties. In particular, the elk1 channel activates very slowly and also deactivates slowly. The elk1 channel shows little or no inactivation during sustained depolarizing voltage steps.

Identification of three new *EAG* genes in addition to the previously known genes and the demonstration that all the *eag* genes are expressed in at least some region of the nervous system raises obvious questions about the function of EAG channels in the mammalian nervous system. At least six *EAG* genes are expressed in sympathetic ganglia, which is a large number of related genes to be expressed in a tissue that contains a relatively homogeneous population of cells. This result suggests that EAG channels are functionally important with, at most, only partially overlapping functions among the different family members. To date, however, there is little convincing data on the physiological function of any of these channels in the mammalian nervous system. In *Drosophila*, mutations in the *eag* and *erg* genes produce hyperexcitability, suggesting a role for these channels in regulating neuronal electrical activity. Ganetzky and Wu, J. Neurogenetics (1983) 1:17-28; Titus et al, J. Neurosci. (1997) 17:85-881; Wang et al, J. Neurosci. (1997) 17:82-890).

Very little information is currently available on the physiological role of elk channels or the related EAG channels in mammals. Both channels have relatively positive activation curves which limit their ability to affect sub-threshold electrical excitability. They activate too slowly to affect the shape of the action potential and, even if they are neuronally expressed, it is difficult to understand how they might affect neuronal excitability. It is possible that heteromultimerization with ß subunits might modify the functional properties of these channels but there is currently no direct evidence to support such a possibility. The *elk*1 gene is expressed in sciatic nerve, which suggests that elk1 channels may function in the glial cells of sympathetic ganglia rather than in neurons.

It has been suggested that EAG subunits co-assemble with more rapidly activating Kv channel subunits to produce heteromers with novel kinetic properties. The *Drosophila* eag channel appears to modify the inactivation properties of the Shaker B channel, possibly by co-assembly into a heteromeric channel (Chen et al, Neuron (1996) 17:35-542). It has yet to be shown, however, that the mammalian Kv channels can be modified by co-assembly with mammalian eag subunits. A recent report of a current expressed in human neuroblastoma cells that has striking similarities to homomeric eag channels expressed in heterologous expression systems (Meyer and Heinemann, J. Physiol. (1998) 508:9-56) suggests that mammalian eag subunits may assemble as independent channels rather than as components of heteromultimeric complexes.

In conclusion, there are a large number of EAG channels expressed in the mammalian nervous system and these channels have a wide diversity of biophysical properties. Surprisingly, the physiological function that any EAG channel

performs in the nervous system has yet to be determined. In the past, the sympathetic nervous system has proven to be a good model system in which to study potassium channels and a large number of different potassium channel currents have been identified (Brown et al, J. Auton. Nerv. Syst. (1982) 6:23-35; Pennefather et al, Proc. Natl. Acad. Sci. (1985) 82:040-3044; Belluzzi and Sacchi, , Prog. Biophys. Molec. Biol. (1991) 55:1-30; Wang and McKinnon, J. Physiol. (1995) 485:19-335). There is, however, a clear excess in the number of potassium channel genes expressed in sympathetic ganglia relative to the number of physiologically identified potassium currents. It will be technically difficult to further refine the electrophysiological techniques necessary to resolve more distinct potassium currents although single channel recordings have provided anecdotal evidence for diversity within apparently homogeneous macroscopic currents. These technical limitations may make it necessary to use genetic approaches such as gene knock-outs to establish the physiological function of the EAG channels in the mammalian nervous system.

The present invention provides the gene sequences for *elk*1, *elk*2 and *eag*2 to enable these genetic approaches for further refining our understanding of the K channels and their functions. The genetic and amino acid sequence information is also useful for the development of treatment methods and pharmaceuticals for the treatment of specific disease conditions. Further the present invention provides methods by which drug candidates can be screened to avoid averse effects on the elk1, elk2 and eag2 potassium channels.

Although the invention has been described with specific embodiments and examples, they are intended to limit the scope of the invention.